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Exhibit H

The Serine Protease Cofactor Factor V Is Synthesized by Lymphocytes[1]

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ABSTRACT. Ag-specific cellular immune responses result in CD4+ T cell activation, which can induce the expression of tissue factor in cells of monocyte/macrophage lineage. This results in initiation of the coagulation protease cascade, with ultimate generation of thrombin. The latter is a potent and pleiotropic mediator of cellular responses and deposition of fibrin. To explore the requirements for extravascular cellular mediation of immune effector pathways, we have searched for a cellular source of the cofactor factor Va. Factor V mRNA was identified in human lymphoid cells by using reverse transcription followed by the polymerase chain reaction (RT-PCR). We confirmed our reverse transcription-polymerase chain reaction results by an independent cloning of factor V cDNA from a T cell cDNA library. The sequence of the factor V cDNA was virtually identical to hepatic factor V mRNA sequence. A limited span of mRNA, encoding part of the connecting region of the factor V protein, was found to contain nucleotide polymorphisms based on six nucleotide substitutions. Northern blot analysis confirmed the presence of a ~7-kb factor V mRNA in the Hut-78* human T lymphoma cell line and, at five- to eightfold less abundance, in unstimulated lymphocytes and long term allogeneic stimulated T cells. Immunocytology with factor V mAb identified factor V intracellularly in freshly isolated T lymphocytes but not on the surface of cells. These data provide evidence for factor V transcription and biosynthesis by human lymphocytes. They provide an additional perspective on how lymphocytes may contribute to inflammatory effector functions of cellular immune responses in extravascular sites through provision of cofactors necessary for the coagulation serine protease cascade. *Journal of Immunology*, 1993, 150: 2992.

Immune effector cell localization and local fibrin deposition are characteristic features of certain types of Ag-driven cellular immune tissue responses reflecting activation of the coagulation protease cascade (1, 2), notably in the classic delayed-type hypersensitivity lesions (3–6). Cells of monocyte lineage express TF[4] through cooperative interactions with Ag-stimulated CD4+ Th cells to initiate protease cascades (7, 8). TF, a cell surface receptor and catalytic cofactor, binds the serine protease factor VIIa, or its precursor zymogen factor VII, either from plasma (9–11) or via biosynthesis by macrophages (12) to form a functional cell surface TF·VIIa complex. This mediates limited proteolytic activation of factor X to the serine pro-

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[4] Abbreviations used in this paper: Va, activated form of factor V; TF, tissue factor; HBS, HEPES buffered saline; RT, reverse transcriptase or transcription; PCR, polymerase chain reaction.

tease factor Xa (9–11). Factor Xa characteristically assembles with the cofactor factor Va^4 [fne^4] for activation of prothrombin to thrombin (13, 14). Tracy et al. (15) observed that mononuclear leukocytes provide a suitable cell surface for the binding of factor Va, factor Xa, and calcium ions to assemble a functional binary complex, Va·Xa, the prothrombinase complex. The product thrombin is a recognized inflammatory mediator eliciting chemotaxis and cellular activation (16, 17) via the thrombin receptor (18), resulting in ion flux and second messenger generation. Thrombin also produces the local fibrin deposition (3–6, 19) characteristically observed in these cellular immune lesions and is associated with a concomitant increase in vascular permeability (19, 20).

Factor V is present in plasma at ~7 μg/ml and is also stored in α-granules of platelets (21). It is a large asymmetric and proteolytically labile glycoprotein (M_r 330,000) that is unlikely to be available and functional in the extracellular fluids. Factor V has latent cofactor function for factor Xa. Upon specific and limited proteolytic activation by thrombin or factor Xa, factor V is converted to factor Va (22, 23). Factor Va, a binding protein and cofactor for factor Xa that associates with cell surfaces, accelerates the rate of thrombin generation from prothrombin by more than 4 orders of magnitude, relative to the free factor Xa alone (13, 14). Factor V cDNA has been cloned from HepG2 cells (24) and human fetal liver (25, 26), and the complete factor V amino acid sequence and genomic organization have been elucidated (27).

There has been a description of factor V-immunoreactive molecules associated with human PBMC populations, appearing to be intracellular (28). A molecule functionally homologous to factor Va, designated as EPR-1, also has been described on the surface of myeloid and lymphoid cells (29, 30). To explore a source of legitimate factor Va cofactor function associated with immune effector cells, we have searched for factor V or similar molecules in human lymphocytes.

Materials and Methods

General methods

All chemicals were of reagent grade. All molecular biology techniques were performed by standard methods (31).

Cells

Peripheral venous blood from normal consenting donors (The Scripps Research Institute, National Institutes of Health General Clinical Research Center, with approval of the Institutional Human Research Committee) was collected in the presence of the anticoagulant heparin or acid citrate dextrose. The platelet-rich plasma was removed from whole blood after centrifugation at room temperature for 15 min at 180 × *g*. The buffy coat was collected and diluted with 2 volumes of RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) and layered over Ficoll-Hypaque (Pharmacia, Piscataway, NJ) to isolate PBMC. Lymphocytes were isolated by centrifugation of PBMC on Sepracell-MN (Sepratech Corp., Oklahoma City, OK). T cells were isolated by nylon wool filtration of isolated lymphocytes with a 60 to 65% yield (32). Platelet contamination was <0.1 platelet/nucleated cell. Allogeneically stimulated T cells were prepared as described previously (30), except that the mixed lymphocyte cultures were stimulated unidirectionally every 7 days by irradiated allogeneic PBMC. Paired frozen human liver tissues and PBMC were obtained from patients with liver cirrhosis that had been referred to the University of Toronto, Toronto, Canada (courtesy of Dr. Gary A. Levy). Liver sections with relatively normal histology were used for isolation of RNA. Hut-78* (a subclone of the T lymphoma cell line Hut-78, ATCC TIB161), MLT, a human leukemia/lymphoma T cell line (a gift from Dr. Deno Dialynas, The Scripps Research Institute), and COS-1, a transformed African green monkey kidney cell line (ATCC CRL1650), were maintained in continuous culture in RPMI 1640 supplemented with 10% FCS (Gemini Bioproducts Inc., Calabasas, CA). HepG2, a human hepatocellular carcinoma cell line (ATCC HB8065), was grown in DMEM supplemented with 1% nonessential amino acids, 1% sodium pyruvate, and 10% FCS.

RNA isolation and Northern blot hybridization

Total cellular RNA was harvested from cell preparations by a guanidinium isothiocyanate-cesium chloride method (31). Poly(A)$^+$ was prepared by passage through an oligo-(dT) column (mRNA isolation kit; Pharmacia). For Northern blot analysis, 20 μg of total RNA were electrophoresed in a 1.0% agarose gel containing 6.6% formaldehyde. After electrophoresis RNA was blotted to nylon membranes (GeneScreen; New England Nuclear, Boston, MA) by capillary transfer in 10× SSPE (1× SSPE is 0.18 mM NaCl, 6.7 mM NaH_2PO_4, 6.7 mM Na_2HPO_4, 1 mM EDTA, pH 8.0). Two hybridization probes were used, 1) a factor V cDNA fragment (nucleotides 5414 to 5904; see *oligonucleotides*) isolated from a plasmid carrying a RT-PCR product, F2/F3, generated from factor V primer pair F2 and F3 (see Fig. 2) and 2) a 350-bp fragment for human GPIIIa (β_3) integrin encoding Ser-97 to Pro-219 (a gift from Joseph Loftus, The Scripps Research Institute). Both probes were labeled with [α-^{32}P]dATP by random priming (Boehringer Mannheim, Indianapolis, IN). Hybridizations were performed at 42°C in 6× SSPE, 1% (w/v) SDS, 10% dextran sulfate (Pharmacia), denatured salmon sperm DNA (100 μg/ml), 50% deionized formamide. Filters were washed twice at 65°C in 2× SSPE/0.5% SDS for 30 min. The final wash was in 0.1× SSPE at room temperature for 1 h. A human β-actin 27-base oligonucleotide (Clontech, Palo

Alto, CA) was labeled with [γ-^{32}P]ATP and used for estimation of this transcript as a control for RNA content on the membrane. Autoradiography of the washed filters was conducted on XAR-5 film (Eastman Kodak, Rochester, NY), by using an intensifying screen, at −70°C. Three to 4 days of development were typical for the factor V probe and platelet GPIIIa probe, with 1 day for the human β-actin probe.

Oligonucleotides

Two sets of 24-base oligonucleotides for RT-PCR were synthesized (Research Genetics, Huntsville, AL) on the basis of the primary sequence of human factor V (25, 26). The sense strands of factor V oligonucleotide primers were designated the "a" series, and the antisense primers were the "b" series. The translational initiation codon ATG was designated as +1. The position of each oligonucleotide is as follows: F1b, 6714 to 6737; F2a, 5881 to 5904; F2b, 5881 to 5904; F3a, 5414 to 5437; F3b, 5441 to 5464; F4a, 4917 to 4940; F4b, 4917 to 4940; F7a, 2776 to 2799; F7b, 2776 to 2799; F8a, 2092 to 2115; F9a, 1573 to 1596; F9b, 1588 to 1611; F10a, 1338 to 1361; F10b, 1330 to 1353; F14a, −25 to −2.

RT-PCR

For RT-PCR, RNA was rendered free of DNA with 0.3 U of DNase I (RNase-free grade; Boehringer Mannheim) at 37°C for 10 min, followed by phenol/chloroform (1:1, v/v) extraction and ethanol precipitation. First-strand cDNA was synthesized with a factor V antisense primer (b series, 0.25 pmol) from total RNA (1 to 2 μg) by using M-MLV RT (BRL, Gaithersburg, MD) or a cDNA synthesis kit (Invitrogen, San Diego, CA). The reactions were stopped by addition of an equal volume of 0.3 N NaOH/0.03 M EDTA, pH 8.0, and heating to 65°C for 1 h, followed by ethanol precipitation. The first-strand factor V cDNA in a mixture of paired factor V oligonucleotide primers (12.5 pmol each) was subjected to PCR amplification (33) by using TaqI (Perkin Elmer Cetus, Norwalk, CT) in a DNA thermocyclic reactor (EpiCom, San Diego, CA). PCR amplification cycles were 94°C for 1 min, 55°C to 63°C for 2 min, and 72°C for 3 min for 25 cycles. Before the first cycle, the reaction mixture was denatured at 94°C for 10 min. After the last cycle, incubation at 72°C was extended for an additional 10 min. An aliquot of the reaction product was electrophoresed through a 2.0% agarose gel, and the DNA bands were visualized by ethidium bromide staining. In some experiments, the RT-PCR reaction product was precipitated with ethanol and digested with *Eco*RI (BRL, Gaithersburg, MD) before agarose gel electrophoresis.

cDNA library and plaque screening

A Hut-78* cDNA library was constructed by priming Hut-78* poly(A)$^+$ RNA (5 μg) with oligo(dT) for the synthesis of cDNA. The cDNA fragments greater than 1 kb were size selected and ligated into a λ gt10 vector modified at the *Eco*RI site by using a Librarian X cloning kit (Invitrogen, San Diego, CA). Plaques (5×10^5) were screened for factor V cDNA clones by using the F2/F3 fragments (Fig. 2), which were radiolabeled by random priming (oligolabeling kit; Pharmacia). Hybridization was performed in the presence of 50% formamide, 10× SSPE, 2× Denhardt's, 0.4% SDS, 100 μg/ml denatured salmon sperm DNA, at 42°C. The final wash of the filters was at 60°C in 0.1× SSC, 0.1% sodium sarcosine. The putative positive λ clones were digested with *Not*I, and the cDNA inserts were isolated, cloned in Bluescript SK2 (Stratagene, San Diego, CA), and isolated for sequencing.

Nucleotide sequencing and analysis

Amplified factor V cDNA fragments produced by RT-PCR were isolated from a 2% agarose gel and purified by using glass beads (GeneClean; Bio 101, San Diego, CA). The cDNA was treated with T4 polynucleotide kinase, blunt-ended with T4 DNA polymerase (New England Biolabs, Boston, MA), and cloned into the *Sma*I site of pUC18 that had been dephosphorylated. Recombinant clones (three to eight) of each RT-PCR product were selected by small-scale plasmid preparation and restriction enzyme digestion (31). Both strands of the factor V insert in each recombinant plasmid were sequenced by the dideoxynucleotide termination method. Sequencing was conducted on an automated DNA sequencer (Applied Biosystems model 370A) by using fluorescently labeled universal sequencing primers. For the nucleotide polymorphism studies, the recombinant plasmid carrying the F7/F8 insert was first digested with restriction enzyme to determine the orientation of insertion at the *Sma*I site. Sequencing was performed on DNA from two or three plasmids with the same orientation, mixed in equal molar ratios. Consensus sequence was obtained from multiple sequencing. The alignments with the hepatic factor V cDNA sequences (25, 26) were performed by using the GenBank data base entry and Intelligenetics programs. The sense strand of HTFV1 and HTFV4 (Fig. 1) were sequenced manually by using Sequenase II (United States Biochemicals, Cleveland, OH).

Production of mAb to human factor V

BALB/c mice were immunized i.p. with 25 μg of human factor V/Va (kindly provided by Dr. Daryl Fair, University of Texas, Tyler, TX, and isolated according to the method of Dahlback (22)) in CFA (0.2 ml), followed by 25-μg boosts of factor V i.p. The spleen cells from mice with a

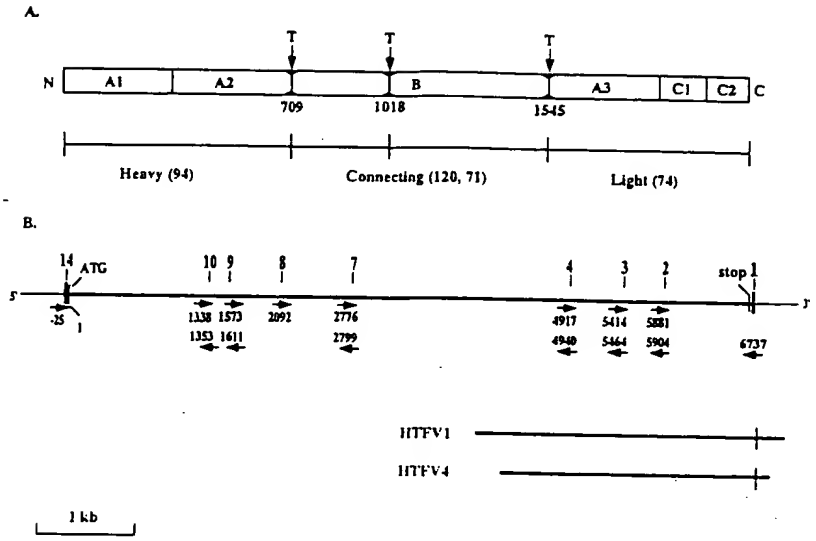

FIGURE 1. Factor V protein and cDNA. *A*, factor V protein (2196 amino acids) consists of three types of homologous domains (*A*, *B*, and *C*). Factor V is activated to factor Va by thrombin (*T*) or factor Xa through limited proteolysis, resulting in a H chain (amino acids 1 to 709, 94 kDa) and L chain (amino acids 1545 to 2196, 74 kDa), which associate by calcium-dependent hydrophobic interactions. *B*, oligonucleotide primers (1 through 14) for RT-PCR reactions are indicated *above the factor V message (7 kb) line. The numbers below the line* are the first nucleotides of each primer. The orientations of sense primers and antisense primers are indicated by the direction of the *arrows*. The translational start site ATG is designed as +1, and *stop* is the termination codon. Factor V cDNA clones (HTFV1 and HTFV4) are indicated.

serum titer of anti-factor V of greater than $1:10^5$ by dot blot were fused with P3×63Ag8.6 myeloma cells. The initial screening of clones producing antibody against factor V was done by solid phase RIA with ^{125}I-labeled V/Va and dot blot analysis with V/Va immobilized on nitrocellulose membranes. Clones producing mAb to factor V/Va were subcloned twice again by limiting dilution. All three selected clones produced IgG1 antibody by isotyping (Zymed Laboratories, San Francisco, CA). The hybridomas were grown in HT medium, 5×10^6 cells were inoculated i.p. into Pristane-primed BALB/c mice, and IgG1 mAb was purified from ascites fluid by using Affigel-protein A (Bio-Rad, Richmond, CA). The anti-factor V mAb 8C4 (IgG1), 15B6 (IgG1), and 22D6 (IgG1) were biotinylated (Calbiochem, San Diego, CA). The mAb that were selected do not neutralize factor Va coagulant activity of plasma and do not react with the monocytic cell line THP-1, in contrast to the EPR-1 mAb (29, 30). By Western blot all three mAb reacted with factor V, intermediate activation products, and purified factor Va L chain but not with H chain (kindly provided by Dr. Kenneth Mann, University of Vermont).

Immunocytology

Immunofluorescence reactions were performed on ice. Freshly isolated T cells were fixed for 5 min in HBS (0.9% sodium chloride, 20 mM HEPES, pH 7.4, 5 mM $CaCl_2$) containing 1% (w/v) paraformaldehyde. After one HBS wash, fixed cells were reacted for 30 min with the designated biotinylated mAb in HBS containing 2.5% BSA, (Sigma Chemical Co., St. Louis, MO) and 0.1% Triton X-100 (Sigma). Cells were washed once and exposed for 30 min to FITC-conjugated streptavidin (Becton Dickinson, San Jose, CA) diluted 1/25 with HBS containing 2.5% BSA. The biotin-conjugated mAb were used at 3 μg/ml final. mAb 22D6, 8C4, and 15B6 were used either mixed in equal amounts in a pool or individually. Anti-human TF mAb IE7 (IgG1) was used as an isotype control. The irrelevant mAb HB3 (IgG2a) to anti-SV40 large T viral Ag served as an additional negative control. Reacted cells were spun onto glass slides (Cytospin; Shandon Southern Instruments, Camberly, Surrey, UK), mounted with Gelmount (Biomeda, Foster City, CA), and observed with a Nikon photomicroscope (Nikon Instruments, San Diego, CA) equipped for epifluorescence. In some experiments, intact peripherial blood-derived T cells were reacted with mAb 22D6, 8C4, and 15B6.

Results

RT-PCR cloning of factor V in Hut-78*

To analyze the presence of factor V transcripts in T cells, we first investigated the Hut-78* cell line. A PCR (33) modified for RNA analysis (RT-PCR) was used to identify putative factor V transcripts. A series of oligonucleotide primers based on the published hepatic factor V cDNA are illustrated in Figure 1. Each antisense b oligonucleotide primer and Hut-78* total RNA were used in the presence of RT to synthesize the first-strand cDNA. Paired primers,

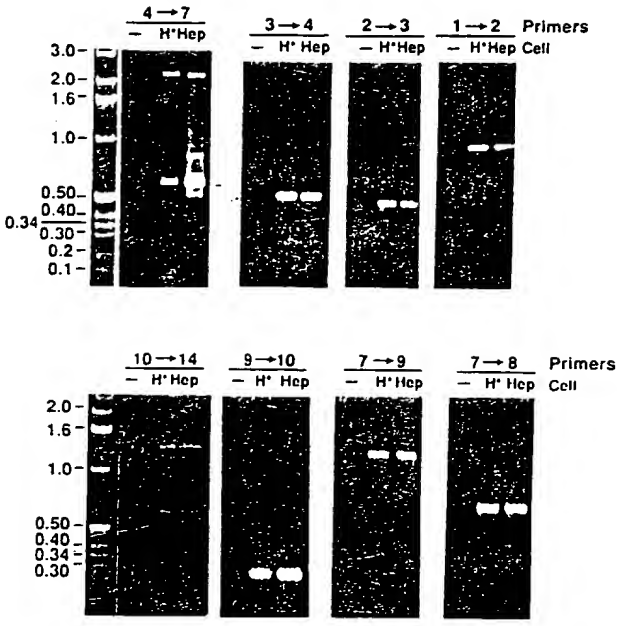

FIGURE 2. RT-PCR-amplified fragments of factor V cDNA. First-strand cDNA synthesis of Hut-78* RNA (1 μg) was either in the presence of RT (*H**) or in the absence of RT (–). HepG2 RNA (1 μg) (*Hep*) was the positive control. The PCR that followed used the indicated pairs of factor V primers. The PCR products (10 μl of a 100-μl reaction) were visualized by ethidium bromide staining of agarose gels. Molecular size markers (1-kb ladder) are shown.

F1 and F2, F2 and F3, and F3 and F4, were used to specifically amplify factor V or related transcripts encoding the L chain region and primer pairs, F4 and F7, F7 and F8, F7 and F9, F9 and F10, and F10 and F14 were used for the amplification of the factor V message encoding the connecting and H chain regions. Exploring conditions of hybridization of varying stringency, we readily observed products of sizes appropriate for factor V that were indistinguishable from those produced by analysis of RNA from the positive control HepG2 cells (Fig. 2). In addition, a 0.6-kb DNA fragment that was shorter than the expected 2.2-kb F4/F7 fragment was observed with RNA from the Hut-78* and HepG2 cells. Sequencing of the 0.6-kb fragment showed sparse sequence similarity with factor V, presumably generated from nonspecific priming of an unrelated transcript in the RT-PCR reactions.

The appropriate size cDNA bands from Hut-78* cells were cloned into pUC18 and individual recombinant plasmids were selected. Multiple clones carrying recombinant plasmids from each segment of amplified factor V cDNA were isolated and the plasmid DNA inserts were sequenced individually. The sequences determined from three to 14 different recloned plasmids provided a consensus sequence for each product. No clones contained deletion or insertion mutations. They agreed with the factor V mRNA sequence from HepG2 and fetal liver cDNA (25, 26), with the exception of the factor V cDNA amplified by using primer pairs F7 and F8.

Both strands from 14 independent recombinant plasmid clones carrying the amplified F7/F8 factor V cDNA fragment were sequenced. Six nucleotide base substitutions were identified (Fig. 3). Two substitutions, from thymine to cytosine and from cytosine to thymine at positions 2209 and 2236, respectively, were silent mutations. The remaining four guanine to adenine base substitutions resulted in a silent mutation at position 2302 and amino acid changes from arginine to lysine at position 2573, from arginine to histidine at position 2595, and from glutamic acid to lysine at position 2773. These deduced amino acid changes are conservative substitutions and may not significantly affect factor V function or only subtly. In addition, half of the clones (seven of 14) have an adenine to guanine substitution at position 2290, a silent substitution, which abolished the *Eco*RI site.

Factor V cDNA library cloning

To confirm and extend the aforementioned observations, a Hut-78* λ gt10 cDNA library was independently constructed and probed with radiolabeled 0.5-kb factor V cDNA fragment F2/F3 generated by RT-PCR from Hut-78* cells. Screening of 5×10^5 recombinant phage gave six positive clones in the primary assay screen. Only the two longest were further characterized; one contained a 3.0-kb insert and the other contained a 2.7-kb insert. These inserts were subcloned in Bluescript to generate plasmids HTFV1 and HTFV4, respectively (Fig. 1). Sequencing of HTFV1 and HTFV4 revealed identity with the hepatic factor V cDNA sequence (25, 26). The translational stop codon and the poly(A) tail were conserved in the Hut-78* factor V transcript, and no putative transmembrane domain sequence was present.

Detection of factor V transcript in lymphocytes

To compare the relative abundance of factor V transcripts in Hut-78* cells with that in HepG2 cells, total RNA was probed with a radiolabeled factor V cDNA fragment F2/F3. As shown in Figure 4*A*, a 7-kb band was detected by Northern blot hybridization, in agreement with the size and abundance of the factor V mRNA from HepG2 cells. The abundance of mRNA was corrected relative to β-actin transcripts.

Further, we inquired whether the expression of factor V mRNA is normal for T cells, rather than an aberrant characteristic of the transformed T cell line Hut-78*. We performed Northern blot hybridization to analyze for factor V transcripts in lymphocytes isolated from healthy individuals. As illustrated in Figure 4*B*, a discrete factor V transcript was identified in RNA from both freshly isolated lymphocytes and long term allogeneically stimulated T

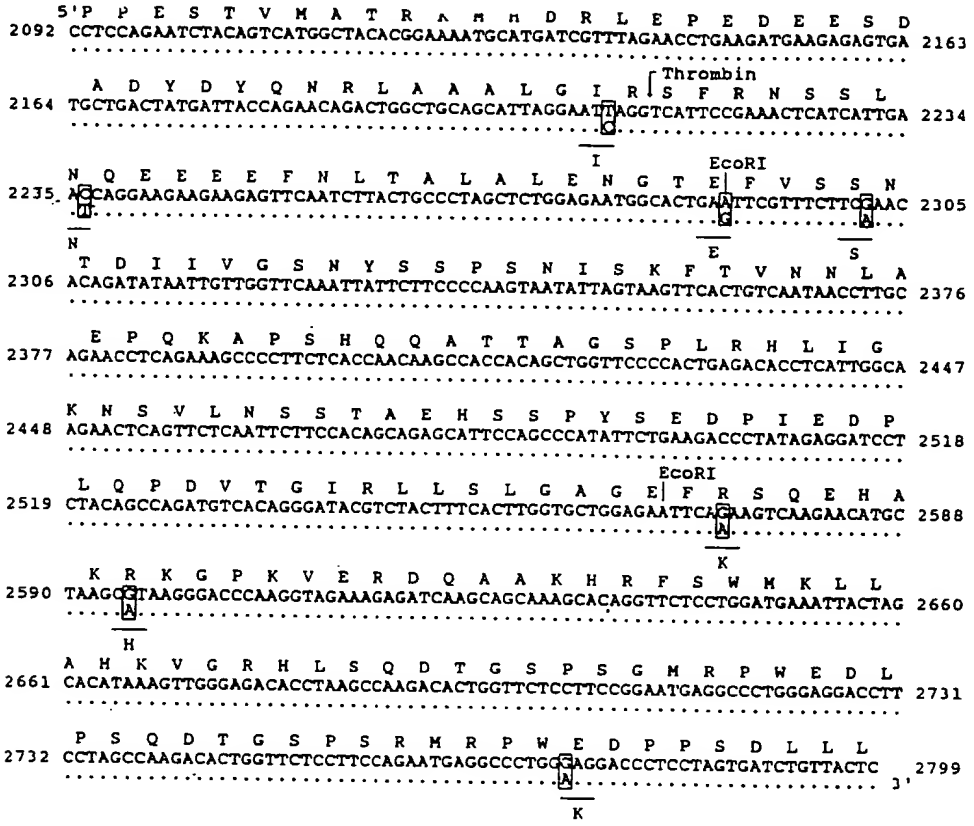

FIGURE 3. Factor V cDNA polymorphisms. Six novel nucleotide substitutions in the span of 2092 to 2799 and one nucleotide mutation at the *Eco*RI site are shown *below* and *boxed* with the published factor V cDNA sequence. The predicated amino acid changes are indicated in single-letter code below the nucleotide substitutions. Sequence differences are only indicated.

cells. It was ~7 kb in length and co-migrated with the major species found in Hut-78* and HepG2 cells. The hybridization signals of lymphocytes and allogeneically stimulated T cells were five- to eightfold less abundant, relative to Hut-78* transcripts, by densitometer scanning. Factor V mRNA could not be identified in the MLT cell line ($CD4^+CD8^+$) or an irrelevant monkey kidney cell line (COS-1), indicating that not all T cell lines or other cells express factor V mRNA. The integrity and amount of RNA samples before hybridization are shown in Figure 4*C*. To rule out platelet contamination as a source of contaminating factor V transcripts, four lines of evidence are available. First, the T cell lymphoma line Hut-78* was positive and cannot be contaminated by platelets. Second, T cells in prolonged in vitro culture with repeated long term allogenic stimulation were positive, which provides evidence for factor V transcripts in nontransformed activated lymphocytes. Third, the Northern blots of lymphocyte RNA (Fig. 4*B*) with radiolabeled probe for GPIIIa, one of the most abundant mRNA species in platelets, gave no signal (data not shown). GPIIIa transcripts have been demonstrated in platelets by RT-PCR (34) and Northern blots (35). Fourth, immunocytologic analysis (see below) demonstrated immunoreactive factor V in most lymphocytes.

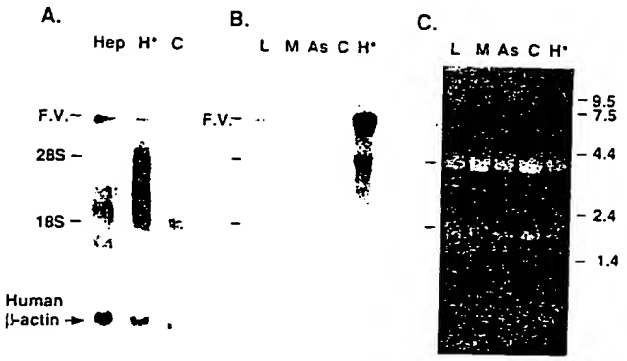

FIGURE 4. Factor V mRNA in T cells by Northern blot. *A*, total RNA (20 μg) from HepG2 (*Hep*), Hut-78* (*H**), and COS-1 (*C*) cells was transferred from a 1.0% formaldehyde-agarose gel to a nylon membrane (GeneScreen) and probed for the presence of factor V transcripts by using radiolabeled factor V cDNA fragment F2/F3. *F.V.*, factor V mRNA (7 kb). The filter was further probed with human β-actin as an internal control, shown at *bottom*. *B*, total RNA (20 μg) isolated from various lymphocyte preparations was probed with a radiolabeled factor V cDNA fragment described in *A*. *Lane L*, lymphocytes; *lane M*, MLT cells; *lane As*, allostimulated T cells; *lane C*, COS-1 cells; *lane H**, Hut-78* cells. *C*, RNA shown in *B* were visualized in a formaldehyde-agarose gel and stained with ethidium bromide before hybridization. The molecular size markers and 28S and 18S rRNA are indicated.

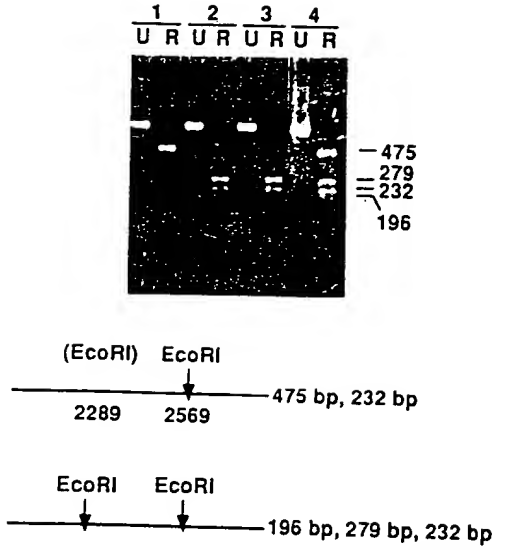

FIGURE 5. *Eco*RI RFLP. Factor V cDNA (F7/F8) fragments were prepared by RT-PCR from lymphocyte RNA (2 μg) of four individuals. It was precipitated with ethanol and divided; half was digested with *Eco*RI (*R*) and half was undigested (*U*). DNA fragments were separated on a 2% agarose gel and stained with ethidium bromide. The sizes of the restriction fragments generated by two *Eco*RI sites are 232 bp, 279 bp, and 196 bp. The *Eco*RI site mutation (in parentheses) at position 2289 resulted in 475-bp and 232-bp fragments.

Nucleotide sequence variation in factor V transcript

Lymphocyte RNA from six healthy individuals was assayed for the novel nucleotide substitutions that were identified in Hut-78*. The amplified RT-PCR products obtained by using primer pairs F7 and F8 were subjected to *Eco*RI enzyme digestion. Figure 5 illustrates three representative patterns of *Eco*RI site polymorphism at position 2289. Three bands, of 279 bp, 232 bp, and 196 bp in length, were identified in individuals having two *Eco*RI sites (Fig. 5, *lanes 2R* and *3R*). Two bands, of 475 bp and 232 bp in length, were found for individuals having only the *Eco*RI site at position 2569, with a loss of the *Eco*RI site at position 2289 (Fig. 5, *lane 1R*). Four bands, of 475 bp, 279 bp, 232 bp, and 196 bp in length, indicated individuals having two types of cDNA, one containing two *Eco*RI sites and the other with only one *Eco*RI site at position 2569 (Fig. 5, *lane 4R*), which may be derived from heterozygote alleles. Sequencing of cloned F7/F8 factor V cDNA from lymphocyte RNA indicated that the six novel nucleotide substitutions (Fig. 3) were observed in five of the six individuals studied, and loss of the *Eco*RI site at nucleotide 2290 is due to an adenine to guanine base substitution. To examine the possibility that these substitutions were lymphocyte-specific post-transcriptional nucleotide modifications, we analyzed paired samples of liver tissue and PBMC from the same individual. The same RT-PCR procedure was applied to two individual samples. Sequencing indicated that the patterns of the six nucleotide substitutions were precisely conserved in RNA from both liver cells and PBMC.

Cellular factor V

The translational product of factor V mRNA was studied in freshly isolated peripheral T lymphocytes. These cells were fixed, either permeabilized or not, and analyzed with specific anti-human factor V mAb (22D6, 8C4, and 15B6) reactive with factor V/Va L chain. Immunofluorescence microscopy illustrates (Fig. 6*C*) that immunoreactive factor V molecules are observed in T cells in condensed interconnecting filamentous structures, suggesting possible concentration in the Golgi, with occasional cells containing high levels of factor V (Fig. 6*D*). There was evident intracellular factor V/Va Ag in approximately 90% of T lymphocytes. Each of the three V/Va mAb gave identical results (data not shown). There were no detectable signals with irrelevant mAb HB3 (IgG2a) or IE7 (IgG1) (Fig. 6*B*). No factor V/Va Ag was detected on the surface of cells (Fig. 6*A*).

Discussion

Serine proteases, including the coagulation, complement, and granzymes are effectors of inflammatory responses locally invoked in Ag-specific immune responses. A central feature of the coagulation and complement cascades is the utilization of protein cofactors for cell surface localization, amplification of catalytic activity, and mediation of specific substrate recognition. The initiating receptor and cofactor TF is induced by Ag-driven Th cells (7, 8) and exhibits these characteristics. However, to propagate the coagulation protease response factor V/Va or a functionally homologous cofactor is required. We established the presence of legitimate transcripts of the precofactor of factor V in T cells. The factor V mRNA is correctly spliced from RT-PCR analysis and of the correct size by Northern blot analysis. There are no deletions, insertions, alternative splicing, or alteration of the translational reading frame determined by sequence analysis. Further, specific mAb localize factor V molecules within the T cells by immunofluorescence microscopy, indicating that the mRNA is translated to protein.

Our data indicate that the abundance of factor V transcripts in freshly isolated lymphocytes is five- to eightfold less than that in Hut-78* T cells or HepG2 cells, consistent with relatively modest levels of expression (Fig. 4). This may explain the failure heretofore to detect factor V transcripts in total PBL by Northern blot analysis (25). Use of RT-PCR permitted direct restriction enzyme digestion and sequence analysis of the factor V transcript of lymphocytes. When various individuals were analyzed, a novel set of nucleotide polymorphisms were demonstrated at the region of the factor V transcript encoding the connecting

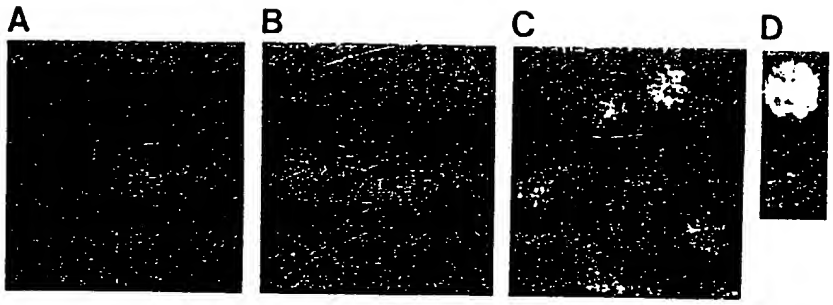

FIGURE 6. Immunocytologic analysis of T cells for factor V. Freshly isolated peripheral blood T cells were reacted with factor V mAb (8C4, 22D6, or 15D6). Either intact cells (*A*) or permeabilized cells (*C*) were used. No reaction was observed for irrelevant mAb (HB3 or IF7) on the permeabilized cells (*B*). *D*, strong reaction for factor V. All panels used 2000× magnification with identical exposure at the same light intensity.

region. Restriction digestion of the RT-PCR product establishes that individuals are either homozygous or heterozygous for an *Eco*RI restriction site. The origin of these nucleotide variations in factor V transcripts is most likely attributed to individual genetic polymorphism, because both lymphocyte and liver from the same individuals had the same patterns of nucleotide changes, excluding the possibility of tissue-specific post-transcriptional nucleotide modifications (36, 37).

Expression of factor V has been established for only limited cell types, including human hepatoma (38), bovine endothelial and smooth muscle cells (39, 40), and rabbit macrophages (41), where it is secreted. Additional studies have demonstrated factor V-immunoreactive molecules that are associated with cells including guinea pig megakaryocytes (42), lysed human PBMC (28), and human megakaryocytes (43). In the present study we identified T cell-associated factor V-immunoreactive molecules by using anti-human factor V mAb that exhibit no detectable cross-reactivity with EPR-1 (30). We found expression of factor V mRNA by Hut-78*, a T lymphoma cell line, which clearly excludes derivation from other cells or factor V endocytosed from plasma. The evolution of the currently known functionally related molecules, including EPR-1, the tumor-associated factor Xa receptor (44), and the recently cloned murine mammary epithelial cell surface molecule (45), remains to be elucidated.

Intracellular sequestration of factor V in T cells not only might provide protection from degradation of this large protease-sensitive protein but also might deliver it to extravascular sites of lymphocyte traffic, similar to delivery of granzymes to the surface of target cells by cytolytic T cells (46). We speculate that mediators involved in T cell activation and collaboration in cellular response may elicit the local release of factor V from lymphocytes. The activation of factor V by factor Xa and formation of the binary Va·Xa prothrombinase complex on local cell surfaces would profoundly enhance local generation of thrombin.

Serine proteases are recognized as potentially important for the effector phase of the immune response. The granzyme family of serine proteases are expressed in thymocytes and activated mature T cells and participate in lytic events by as yet obscure mechanisms (47, 48). Further, evidence exists that serine protease activity may be required for optimal lymphokine release by T cells (49). These observations, coupled with primary sequence homology between certain T cell serine proteases and coagulation serine proteases (50), lead to a unifying hypothesis. Because the coagulation protease cascade utilizes cofactors for cell surface localization, enhancement, and regulation of functional proteolytic activity, the possibility should be considered that the participation of cofactors for cell surface assembly of cellular serine proteases may represent a general paradigm.

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